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Exploring the biocatalytic scope of a bacterial flavin-containing monooxygenase†

Ana Rioz-Martínez,[a] Malgorzata Kopacz,[b] Gonzalo de Gonzalo,*[b] Daniel E. Torres Pazmiño,[b] Vicente Gotor[a] and Marco W. Fraaije*[b]

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A bacterial flavin-containing monooxygenase (FMO), fused to phosphite dehydrogenase, has been used to explore its biocatalytic potential. The bifunctional biocatalyst could be expressed in high amounts in *Escherichia coli* and was able to oxidize indole and indole derivatives into a variety of indigo compounds. The monooxygenase also performs the sulfoxidation of a wide range of prochiral sulfides, showing moderate to good enantioselectivities in forming chiral sulfoxides.

Introduction

Flavin containing monooxygenases (FMO) (E.C. 1.14.13.8) represent single-component flavoprotein monooxygenases that contain a tightly bound FAD cofactor.[1] In their catalytic cycle, the flavin cofactor is reduced by NADPH after which it reacts with molecular oxygen to form a hydroperoxyflavin intermediate.[2] This reactive enzyme species is able to perform oxygenation of heteroatom-containing compounds that are able to reach its active site. In humans, five FMO isoforms are present that show a tissue-specific distribution.[3] The role of these oxidative enzymes is thought to be similar to that of most human cytochrome P450 monooxygenases: detoxification of drugs and other xenobiotics into more hydrophilic metabolites.[4] The crucial detoxifying role of human FMOs becomes apparent when considering trimethylaminuria, also known as fish-odour syndrome. This metabolic disorder is caused by mutations in human FMO3 and results in accumulation of trimethylamine, causing a "fish odour".[5]

Most of the known FMOs have been shown to be membrane-associated, prohibiting facile isolation. As a result, FMOs have rarely been studied for biocatalytic purposes.[6] Some years ago the first bacterial FMO, mFMO from *Methylophaga* sp. strain SK1, was reported which was shown to be a soluble enzyme that could be overexpressed in *E. coli*.[7] In fact, inspection of sequenced bacterial genomes with the aid of an FMO-specific sequence motif has revealed a large number of genes that putatively encode soluble FMOs.[8] Therefore it is attractive to start exploring this class of monooxygenases.

Little biocatalytic data on mFMO have been reported. mFMO is a homodimer with subunits of 54 kDa, showing around 30% sequence identity with the five human FMOs.[7] mFMO was shown to be able to convert endogenous indole in *E. coli* cells and this capacity has been optimized to form up to 920 mg indigo blue per litre of fermentation broth.[9] It was also shown that the bacterial enzyme is able to convert similar substrates when compared with human FMOs, *e.g.* trimethylamine and (*S*)-(−)-nicotine are efficiently oxidized by mFMO.

Recently we have described the preparation of self-sufficient monooxygenases by covalent coupling of Baeyer–Villiger monooxygenases (BVMOs) with the soluble NADPH-regenerating phosphite dehydrogenase (PTDH) from *Pseudomonas stutzeri*.[10] These bifunctional biocatalysts are able to use phosphite as a cheap and sacrificial substrate for recycling NADPH. An improved expression vector for producing these bifunctional biocatalysts has been developed using a codon-optimized gene encoding a His-tagged and thermostable PTDH mutant as fusion partner.[11]

For this exploratory biocatalytic study on mFMO, we have used this newly developed expression vector. The produced self-sufficient mFMO was explored for the preparation of chiral sulfoxides, target molecules with a high interest due to their biological properties and widespread applications in organic synthesis.[12] Furthermore, we looked into the applicability of mFMO for the preparation of indigo derivatives, valuable as dyes or precursors for pharmaceuticals,[13] which have been targeted before with other monooxygenases with varying degrees of success.[14]

Results and discussion

The fused PTDH-mFMO could be readily expressed as soluble and bifunctional enzyme at high levels. By a one-step purification, 102 mg of pure and soluble PTDH-mFMO could be obtained from 1.0 L of culture broth. The bifunctional biocatalyst was tested for activity at several temperatures with trimethylamine. This revealed that the optimal temperature for activity is 70 °C. However, at this temperature the enzyme is quickly inactivated with a total loss

[a]*Departamento de Química Orgánica e Inorgánica, Instituto Universitario de Biotecnología de Asturias, Universidad de Oviedo, C/Julián Clavería 8, 33006, Oviedo, Spain*

[b]*Laboratory of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands. E-mail: g.de.gonzalo.calvo@rug.nl, m.w.fraaije@rug.nl; Fax: +31 50 363 4165; Tel: +31 50 363 4345*

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of activity after 2 min. Analysis of the thermostability at 35 °C revealed a half-life of 5 h for mFMO, without affecting the activity of PTDH. For further experiments, it was decided to perform conversions at 25 °C. It was also established that the enzyme was most effective at pH 9.0 (see Supporting Information†).

It is known that mFMO is able to form indigo blue from indole.[7] This was also clearly visible when growing cells for PTDH-mFMO overproduction: the growth medium turned dark blue. To establish whether mFMO can also be used for synthesis of other indigo derivatives, isolated PTDH-mFMO was incubated with a range of indole derivatives. Visual inspection revealed that all tested indoles were accepted as substrate as evidenced by formation of a variety of colors (Fig. 1). By steady-state kinetic analysis the reaction rates for all indole derivatives were determined (see Supporting Information). Indole was found to be converted most efficiently.

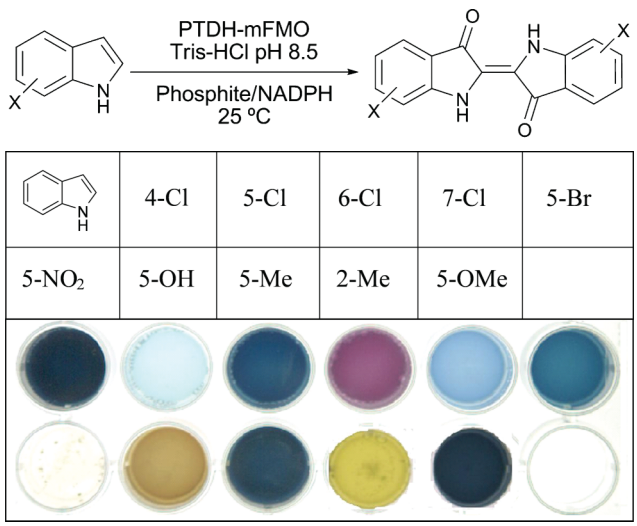


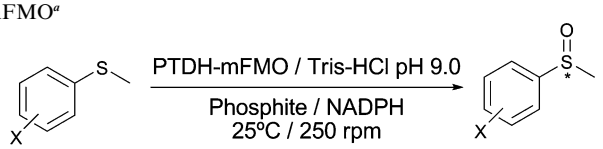
Fig. 1 PTDH-mFMO catalyzed oxidation of indole and indole derivatives into indigoid dyes.

As mFMO has been shown to convert indoles, a set of substituted aromatic sulfides was tested as substrates. Most of the tested sulfides were shown to be oxidized and exhibited quite good K_M values, ranging from 100 to 600 μM, while the k_{cat} values were only modest: ~0.5 s^{-1} (see Supporting Information†). PTDH-mFMO was subsequently assayed for enantioselective oxidation of thioanisole derivatives (Table 1). Reactions were performed in Tris-HCl buffer which contained 1% DMSO in order to dissolve the sulfides. In most cases, the (*S*)-sulfoxides were formed in excess.

The most effective conversion was found with thioanisole **1a**, yielding 95% of (*S*)-methyl phenyl sulfoxide **1b** after 8 h, albeit with a moderate enantiomeric excess (ee = 35%). Substituents in the aromatic ring decreased the enzymatic activity. PTDH-mFMO was able to oxidize methyl *p*-tolyl sulfide (**4a**) and *p*-chlorophenyl methyl sulfide (**5a**) into the corresponding sulfoxides (*S*)-**4**–**5b** with high enantiomeric excesses (ee>90%). Good enantioselectivities were also obtained in the oxidation of the *p*-hydroxy (**2a**), *p*-methoxy (**3a**) and *o*-chloro (**7a**) derivatives, while the chloro-substitution in the *meta*-position (**6a**) as well as the presence of electron-withdrawing groups (**8**–**10a**) had a negative effect on the selectivity of the formed sulfoxide.

As well as oxidation of thioanisole derivatives, some other aromatic sulfides were also tested. As indicated in Table 2, the

Table 1 Sulfoxidation of thioanisole derivatives catalyzed by PTDH-mFMO[a]



1-10a (*R*)- or (*S*)-**1-10b**

Sulfide	X	Time (h)	Conv. (%)[b]	ee (%)[c]
1a	H	8	95	35 (*S*)
2a	*p*-OH	15	37	81 (*S*)
3a	*p*-OMe	15	78	70 (*S*)
4a	*p*-Me	15	66	92 (*S*)
5a	*p*-Cl	15	80	95 (*S*)
6a	*m*-Cl	15	69	15 (*R*)
7a	*o*-Cl	15	81	75 (*R*)
8a	*p*-COMe	15	8	21 (*R*)
9a	*p*-CN	15	50	22 (*S*)
10a	*p*-NO_2	15	47	37 (*S*)

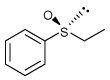
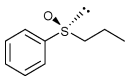
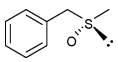
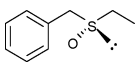
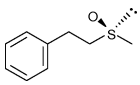
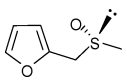
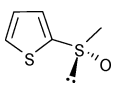
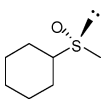
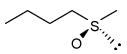
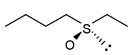
[a] For reaction details, see Experimental. [b] Measured by GC. [c] Enantiomeric excesses measured by HPLC.

oxidation of phenyl ethyl sulfide (**11a**) and its propyl analogue (**12a**) led to a significant loss in conversion when compared to thioanisole, but the corresponding (*S*)-sulfoxides can be obtained with much better enantiomeric excess (ee = 79%). (*S*)-Benzyl methyl sulfoxide (*S*)-**13b** and the ethyl derivative **14b** were formed with excellent or good conversions, respectively, while the biocatalyst showed a poor enantioselectivity for these compounds. When the sulfur atom was further away from the phenyl moiety (**15a**) a good enzymatic activity and moderate enantioselectivity was observed. No oxidation was achieved for those sulfides presenting one (methyl naphthyl sulfide, **16a**) or two bulky substituents (benzyl phenyl sulfide, **17a**). PTDH-mFMO has also been tested in the synthesis of heteroaromatic chiral sulfoxides, important chiral auxiliaries in asymmetric catalysis. Methyl 2-pyridyl sulfide, 2-furfurylmethyl methyl sulfide and 2-(methylthio)thiophene were converted into the corresponding sulfoxides with high conversions (around 80%). (*S*)-**18b** and (*S*)-**20b** were obtained with moderate enantiomeric excess, while the formation of (*R*)-**l9b** occurred with a low selectivity.

As mFMO has been shown to be active on aliphatic amines, PTDH-mFMO was also tested with several aliphatic sulfides. This has revealed that it displays a relatively low activity and selectivity for the oxidation of cycloalkyl alkyl sulfides (**21a**). Sulfoxidation of tetrahydro-2*H*-thiopyran (**22a**) did not take place, even after long reaction times. Finally, we focused on the biooxidation of linear aliphatic sulfides. It was found that alkyl butyl sulfides **23a** and **24a** were oxidized with moderate conversion and good enantiomeric excesses in order to obtain (*R*)-**23b** and (*R*)-**24b**, respectively. The presence of a longer alkyl chain in the sulfide structure had a negative effect on the reactivity, as no reaction was observed in the oxidation of *n*-octyl methyl sulfide **25a**.

PTDH-mFMO was also tested in the oxidation of racemic sulfoxides. Incubating racemic methyl phenyl sulfoxide (±)-**1b** with this biocatalyst led to a moderate conversion, with only 8% of the final sulfone while **1b** remained racemic. No sulfoxide oxidation was observed for compounds (±)-**4b**, (±)-**5b** and (±)-**7b** after long reaction times.

Table 2 PTDH-mFMO catalyzed synthesis of chiral sulfoxides starting from the corresponding sulfide[a]

Sulfide	Sulfoxide	Time (h)	Conv. (%)[b]	ee (%)[c]
11a		15	15	79 (*S*)
12a		24	12	71 (*S*)
13a		8	91	17 (*S*)
14a		8	75	15 (*S*)
15a		15	80	36 (*S*)
18a		15	83	49 (*S*)
19a		15	72	20 (*R*)
20a		15	76	50 (*S*)
21a		15	14	20 (*R*)
23a		15	52	85 (*R*)
24a		15	71	77 (*R*)

[a] For reaction details, see Experimental. [b] Measured by GC. [c] Enantiomeric excesses measured by HPLC.

The close homology of FMOs and BVMOs[1] inspired us to test substrates that are readily oxidized by the latter type of enzymes. However, after long reaction times, no product formation was observed in the presence of different ketones (2-octanone, cyclohexanone and acetophenone) or phenylboronic acid.

In a previous report, the influence of the organic cosolvents on the oxidation of prochiral sulfides catalyzed by BVMOs has been described.[15] Short alkyl chain alcohols as cosolvents were shown to result in an increase or even a reversal in enantioselectivity for some BVMOs. To probe this effect on mFMO, oxidation of **1a** was performed in the presence of several organic cosolvents (5% v/v) with different physico-chemical properties. All the cosolvents tested led to lower conversions and/or enantiomeric excesses (see Supporting Information†), with the exception of the reaction in 5% hexane. For this cosolvent, (*S*)-**1b** was recovered with a 48% ee and 62% extent, while 28% of methyl phenyl sulfone **1c** was formed. Sulfoxidation with a combination of 1% DMSO and 5% hexane occurred only with 24% conversion and 34% ee for (*S*)-**1b**. From the data it becomes clear that 5% hexane has an activating effect on the oxidation of the sulfoxide **1b**, while the presence of DMSO in the reaction medium inhibits sulfoxide oxidation, as can be shown in the experiments developed by employing PTDH-mFMO in the biooxidation of (±)-**1b** (entries 6–8, Table 3). Thus, the increase in the enantiomeric excess observed when working in 5% hexane is due to the desymmetrization of prochiral **1a** combined with the kinetic resolution of **1b**. *n*-Hexane presents a somewhat similar structure to 1-octylamine, which has been described as a specific activator in human FMOs.[16] The presence of 3 mM of this additive led to a higher conversion while it did not significantly affect the enantioselectivity (see entry 5).

Table 3 Effect of hexane in the sulfoxidation of **1a** and (±)-**1b**[a]

Entry	Compound	Cosolvent	Conv. (%)[b]	ee (%)[c]
1	**1a**	None	48 (2)	33
2	**1a**	1% DMSO	95 (5)	35
3	**1a**	5% Hexane	90 (28)	48
4	**1a**	5% Hexane 1% DMSO	24	34
5	**1a**	3 mM $OctNH_2$	76 (7)	27
6	(±)-**1b**	1% DMSO	8	5
7	(±)-**1b**	5% Hexane	61	72
8	(±)-**1b**	5% Hexane 1% DMSO	9	5

[a] Reaction time 8 h. For other reaction details, see Experimental. [b] Measured by GC. Amount of sulfone in brackets. [c] Measured by HPLC.

In order to show that this bifunctional enzyme can be used as an effective biocatalyst, we set up a semipreparative experiment, in which sulfide **5a** was used as model substrate. Sulfoxidation was performed by incubating 100 mg of prochiral **5a** in the presence of PTDH-mFMO (4.0 μM), sodium phosphite as cosubstrate, NADPH (0.2 mM) and dioxygen as oxidant. After 24 h, (*S*)-**5b** was achieved with a 90% conversion and 95% enantiomeric excess. The sulfoxide product was isolated by column chromatography, to afford 77% isolated yield.

Conclusions

A novel fused oxidative biocatalyst has been overexpressed in *E. coli*, isolated and purified. PTDH-mFMO was found to be able to oxidize indole and analogues into the corresponding indigoid pigments which represent interesting dyes and bioactive compounds. The bifunctional dehydrogenase-FMO system has been applied for the first time in the biocatalysed sulfoxidation of prochiral sulfides. Depending on the substrate structure, excellent enantioselectivities can be achieved. The data show that bacterial FMOs represent an unexplored and interesting class of oxidative biocatalysts which may be of use for synthetic purposes. The availability of the crystal structure of mFMO[2a] can also facilitate redesign of FMOs for specific biocatalytic applications.

Experimental

Recombinant PTDH-mFMO was overexpressed and purified following the previously described procedure.[10] 1.0 Unit of PTDH-mFMO will oxidize 1.0 μmol of thioanisole to methyl phenyl sulfoxide per minute at pH 9.0 and room temperature in the presence of NADPH. Starting prochiral sulfides **1a**, **6a**, **8a** were

purchased from Sigma–Aldrich–Fluka, **2–5a**, **7a**, **9–10a**, **11a**, **16a**, **17a**, **19a**, **20a** and **21a** were supplied by Alfa Aesar, **13a**, **22a**, **23a** and **24a** were obtained from Acros Organics and compound **25a** was a product by TCI Europe. All the starting indoles were products from Sigma-Aldrich-Fluka, with the exception of 4-chloroindole, which was purchased from Acros Organics. All other reagents and solvents were of the highest quality grade available.

Chemical reactions were monitored by analytical TLC, performed on silica gel 60 F254 plates and visualized by UV irradiation. Flash chromatography was carried out with silica gel 60 (230–240 mesh). Kinetic parameters were measured in a Varian Cary50Bio UV/Vis spectrophotometer. Melting points were taken on samples in open capillary tubes and are uncorrected. IR spectra were recorded on infrared spectrophotometer using KBr pellets. ^{1}H-NMR, ^{13}C-NMR and DEPT spectra were recorded with tetramethylsilane (TMS) as the internal standard with a DPX (^{1}H: 300.13 MHz; ^{13}C: 75.5 MHz) spectrometer. The chemical shift values (δ) are given in ppm. Optical rotations were measured using a polarimeter and are quoted in units of 10^{-1} deg cm^2 g^{-1}. APCI$^+$ and ESI$^+$ using a chromatograph mass detector or EI$^+$ with a mass spectrometer were used to record mass spectra (MS). High-resolution mass spectra were obtained with a Bruker Microtof-Q-spectrometer.

Methyl phenylethyl sulfide **15a** was synthesized by treating at 0 °C the corresponding thiol with sodium and methyl iodide in dry MeOH, under a nitrogen atmosphere (40% yield).[17] 2-(Methylthio)pyridine **18a** was prepared by reaction of the corresponding thiol and potassium carbonate with iodomethane and triethylamine in CH_2Cl_2 at 0 °C (99% yield).[18] *p*-Acetoxyphenyl methyl sulfide **2c** was obtained from **2a** by acetylation in presence of acetic anhydride and pyridine (90% yield). Racemic sulfoxides (±)-**1–15b**, (±)-**18–21b** and (±)-**23–24b** were prepared by chemical oxidation of the corresponding sulfides employing hydrogen peroxide and methanol (yields higher than 60%). All the synthesised compounds **15a**,[19a] **18a**,[18] **1b**,[17] **2b**,[19b] **3–7b**,[17] **8b**,[19c] **9–15b**,[17] **18b**,[19d] **19b**,[19e] **20b**,[19f] **21b**,[19d] **23b**,[19g] **24b**[19d] exhibited physical and spectral data in agreement with those reported.

Absolute configurations of sulfoxides **1b**,[17] **3–7b**,[17] **9–15b**,[17] **18b**,[20a] **19b**,[20b] **20b**,[20a] **21b**[19e] were determined by comparison of elution order on HPLC with published data, meanwhile the absolute configurations of sulfoxide **2b** and **8b** were established by comparing the retention times on HPLC for acetylated **2b** and **8b** with the ones obtained in the asymmetric sulfoxidations of prochiral sulfides **2c** and **8a** employing (+)-diethyl L-tartrate, Ti(*O*-*i*-Pr)$_4$ and TBHP.[21] The absolute configuration of compound **23b** was obtained by comparing the specific rotation value with published data,[22] while absolute configuration of butyl ethyl sulfoxide **24b** was established by analogy with this latest one.

General procedure for the oxidation of indole and derivatives employing PTDH-mFMO

A plate with 24 wells was employed. A solution of the corresponding starting indoles (5.0 mM) was dissolved in Tris-HCl buffer 50 mM, 35 mM NaCl, pH 8.5 containing sodium phosphite (10 mM), NADPH (0.2 mM) and self-sufficient biocatalyst PTDH-mFMO (4 μM). Reactions were stirred at 25 °C for 18 h and products formation was observed colorimetrically.

General procedure for the PTDH-mFMO biocatalysed sulfoxidations

Unless otherwise stated, prochiral sulfides **1**–**25a** (5 mM) were dissolved in a 50 mM Tris-HCl buffer pH 9.0 (1.0 mL) containing 1% DMSO, sodium phosphite (10 mM), NADPH (0.2 mM) and the self-sufficient biocatalyst PTDH-mFMO (4 μM). Reactions were stirred at 25 °C and 250 rpm for the times established. Reactions were then stopped, extracted with EtOAc (2 × 0.5 mL), dried onto $MgSO_4$ and analyzed by GC and/or HPLC in order to determine the conversions and the enantiomeric excesses of the sulfoxides (*R*)- or (*S*)-**1–25b**. Control reactions in absence of biocatalyst were performed and did not result in any conversion.

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